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REVIEW ARTICLE

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Origins of a pervasive, erroneous idea: The "green birefringence" of Congo red-stained amyloid

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Summary

Congo red was discovered to stain amyloid by accident in 1922, and Congo red-stained amyloid was shown to be birefringent on polarization microscopy in 1927. Colours, namely green and yellow, were reported under these conditions in 1945, although these are only two of various anomalous colours that may be seen, depending on the optical set-up. In 1953 there began a dogmatic insistence that in Congo red-stained amyloid between crossed polarizer and analyser green alone should be seen, and the finding of any other colour was a mistake. The idea that green, and only green, is essential for the diagnosis of amyloid has persisted almost universally, and virtually all mentions of Congo red-stained amyloid say that it just shows "green birefringence" or "apple-green birefringence." This idea is wrong and is contrary to everyday experience, because green is seldom seen on its own under these conditions of microscopy, and often, there is no green at all. How observers maintain this unscientific position is explained by a study of its historical origins. Most of the early literature was in German or French and was usually quoted in English at second hand, which meant that misquotations, misattributions and misunderstandings were common. Few workers reported their findings accurately, hardly any attempted to explain them, and until 2008, none gave a completely satisfactory account of the physical optics. The history of Congo red-stained amyloid is an instructive example of how an erroneous belief can become widely established even when it is contradicted by simple experience.

KEYWORDS

amyloid, anomalous colours, Congo red, polarization microscopy

1 | INTRODUCTION: CONGO RED AS A STAIN FOR AMYLOID

Scientific evidence for the accuracy and usefulness of clinical investigations is important in medicine. Despite this, there is still a striking example of a demonstrably unscientific and widespread belief in what should be found in a diagnostic test for amyloidosis. How this belief arose and the history of ideas about the test are the subjects of this review.

Amyloidosis is a group of conditions in which misfolded proteins of various types are deposited in tissues as fibrils. These amyloid fibrils bind the dye Congo red, and this is used as a diagnostic test on microscopy.[1] When a stained section is examined by polarization microscopy, specifically when

polarizing filters, a polarizer and an analyser, are inserted in the light path below and above the section, various colours can be seen, depending on the degree of rotation of one filter compared with the other. These filters convert ordinary, unpolarized light into light that only travels in one plane, called linearly polarized light, and when they are accurately crossed, the background is dark, but birefringent materials appear bright. Almost invariably, Congo red-stained amyloid is only reported to show "green birefringence" or "apple-green birefringence" under these conditions. This is said to be diagnostic of amyloid and is almost universally reported as the evidence of amyloid.[2]

In everyday medical practice, which is mirrored by colour illustrations in papers, green is unlikely to be seen on its own, even when the polarizer and analyser are accurately crossed. The microscopic optics must be perfect to show pure green. If the optics are not perfect, which is almost inevitable on most microscopes, a mixture of colours is seen, typically blue-green and yellow-green, which can be called green and yellow, or even definite blue and yellow, without any green at all (Figures 1 and 2). If two colours are seen, they exchange positions when the section is rotated by 90° on the microscope stage. If the polarizer and analyser are not accurately crossed, mixtures of colours are always seen, and these vary depending on the extent of uncrossing of the filters, and whether initially there is only green or a mixture of colours. Examples of colours seen as the filters are uncrossed are light blue-green and orange, and reddish-purple and lemon yellow. In sections thicker than usual, even in perfect conditions, yellow, orange or red are seen, rather than green.[2-5]

These colours can be seen by anyone with a suitable microscope. The same findings would have been evident from the time of the earliest interest in the optical properties of Congo red, because polarization microscopy was well-developed by then.[6] The first aim of this paper is to trace the development of correct and incorrect ideas about the physical optics that explains the colours, which requires brief accounts of the relevant principles to allow them to be understood without any specialized knowledge of physics and without the need to refer to other papers or texts. The second aim is to find out how there is the insistence on green, and green alone, in the diagnosis of amyloid, including when, as often occurs, no green at all is seen.[2-5]

2 | THE DISCOVERY THAT CONGO RED STAINS AMYLOID

The abnormal human condition now called amyloidosis has been known for a long time, initially under various names, such as lardaceous or waxy change.[7] In 1854, the deposited material was given the name amyloid, literally meaning starch-like, by the great German pathologist Rudolf Ludwig Karl Virchow (1821-1902).[8-10] Despite a common idea, this was not because he thought the human material resembled starch. "Amyloid" had been first used by the German botanists Julius Rudolf Theodore Vogel (1812-1841) and Matthias Jakob Schleiden (1804-1881) in a paper in 1839, although their work had been done in 1838.[11] They invented amyloid as a name for a plant material that to acquire a property of starch, namely, turning

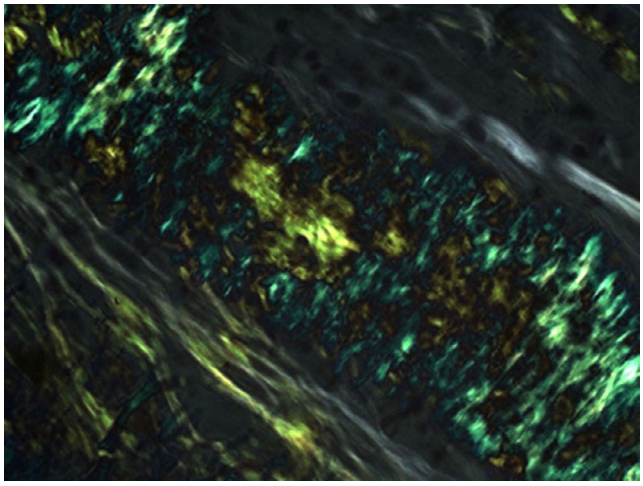

FIGURE 1 Amyloid in a kidney stained by Congo red, examined between crossed polarizer and analyser. Green and yellow, or blue-green and yellow-green, are seen. These anomalous colours are a typical finding in everyday practice. This is representative of the kind of image reported to show only "green birefringence" or "apple-green birefringence." Reproduced from *Bull R C Pathol*. **144**, 263-266 (2008), with permission

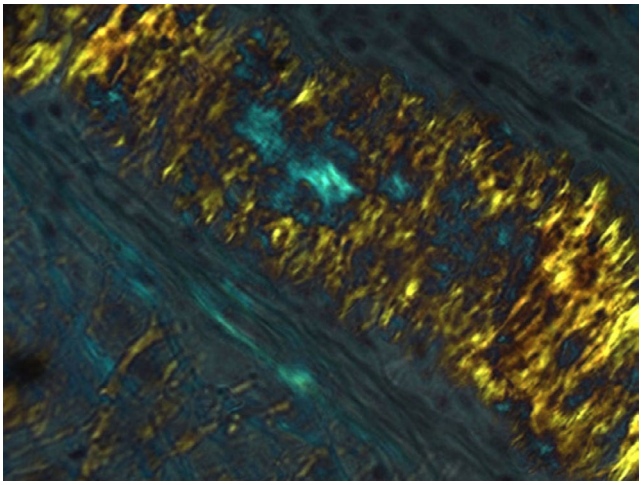

FIGURE 2 The field in Figure 1 after optical manipulation with an elliptical compensator to give yellow and blue, after complete compensation of blue and yellow, respectively. Just as in Figure 1, these anomalous colours are often found and are reported to show only "green birefringence" or "apple-green birefringence." Reproduced from *Bull R C Pathol*. **144**, 263-266 (2008), with permission

blue on addition of iodine, had to be treated with sulphuric acid. The plant material was in fact cellulose, which was first recognized by a French industrial chemist, Anselme Payen (1795-1871), also in 1838,[12] although the name cellulose was actually invented by three French scientists commenting on Payen's work, the botanist Adolphe-Théodore Brongniart (1801-1876), the chemist Théophile-Jules Pelouze (1807-1867) and the chemist Jean Baptiste André Dumas (1800-1884).[13-15] Virchow found that the human material resembled cellulose rather than starch, but was reluctant to call it cellulose, and suggested amyloid as a compromise, because botanists no longer used that term.

Congo red was a product of the German synthetic dye industry.[16,17] Dyes appear coloured because they absorb light of certain wavelengths, and their colour is white minus the absorbed wavelengths. Congo red absorbs blue and green wavelengths, with a peak in the blue-green, and so appears red in ordinary illumination. Its value as a histological stain was reported in 1886 soon after its commercial introduction in 1885, although there was no mention then of its use on amyloid.[18]

Staining of amyloid by Congo red was discovered by chance in 1922. A physician in Hamburg, Hans Hermann Bennhold (1893-1976), was investigating an established clinical test to measure blood volume, which was intravenous injection of a solution of Congo red followed by study of its concentration in the blood at intervals after the injection. Although he said in his German publications at the time that he did this to find out what happened to the Congo red, in fact he revealed much later that he was investigating the potential value of the test as an indicator of liver function.[19-24] Autopsy on a patient who died twenty hours after the injection showed areas in the liver, spleen and kidneys that were red in unstained frozen sections and corresponded with the sites of deposition of amyloid shown by a conventional staining method at that time, which was detection of metachromasia with methyl violet. Metachromasia is a change of colour in ordinary illumination. This method had replaced the earlier iodine-sulphuric acid method.[25,26]

Bennhold then found that Congo red disappeared from the blood faster in patients with amyloidosis than in other patients. Bennhold's intravenous Congo red test became a standard clinical investigation for amyloidosis until the late 1960s, while staining of sections with Congo red came into routine practice, but only slowly. Until the late 1960s, metachromatic dyes, usually crystal violet or methyl violet, were still commonly used to diagnose amyloid.[27-29] One reason was that Bennhold's staining method was difficult and often detected materials other than amyloid. Later improvements in the method, published in English, gradually helped to popularize Congo red, especially those made by Highman in 1946, Puchtler, Sweat and Levine in 1962, and Stokes in 1976.[30-32]

3 | EARLY POLARIZATION MICROSCOPIC OBSERVATIONS

In 1888, the German botanist and microscopist Ernst Ludwig Victor Hermann Ambronn (1856-1927) reported that Congo red molecules had an orderly arrangement on plant cell walls, which consist of cellulose.[33] This arrangement showed itself because the dye was seen to be dichroic, which means that the molecules were orientated in such a way that they absorbed light polarized in one plane, and so appeared dark red, much more than light polarized in the plane at right angles, when they appeared light red. To detect dichroism, only one polarizing filter is required, either a polarizer or an analyser. Rotation of the filter or the microscopic preparation allows the dichroism of Congo red to be detected, because the dye absorbs light only when the light is polarized parallel to its light-absorbing atomic bonds. This was the beginning of polarization microscopic study of Congo red, although only its property of dichroism was found then.

Despite claims that Bennhold reported the birefringence of Congo red-stained amyloid, and even a green colour, he did not, because he did not use polarization microscopy.[34-36] Birefringence means that a transparent material transmits light at different velocities depending on the orientation of the material in relation to the plane of linearly polarized light. The refractive index is the ratio of the velocity of light in air or a vacuum to the velocity in a material. A birefringent material has two extremes of refractive index, because light travels most slowly through it in one plane, called the slow axis, with the largest refractive index, and least slowly through the plane at right angles to this, called the fast axis, with the smallest refractive index. There is a range of refractive indices between these limits.

Birefringence and the explanation of the brightness of birefringent materials had been known for a long time.[37] When any birefringent material has the fast and slow axes at 45° to the plane of linearly polarized light, which is the optimal position to detect birefringence, the light usually becomes elliptically polarized, which means that the tip of a vibrating light wave leaving the material rotates and traces an elliptical path, rather than just vibrates in a straight line in the plane of the polarizer. This is because the light can be considered to be resolved into two vectors perpendicular to each other, one in the fast axis and one in the slow axis. These take a different time to pass through the material, and on reaching air, they recombine into one elliptically polarized wave. Some light can pass a crossed analyser, and the material appears bright (Figure 3).

The birefringence of orientated Congo red, although not of Congo red-stained amyloid, was reported in German in 1925 by the microscopist Hans Neubert, and his findings were expanded later, also in German, by the Swiss industrial

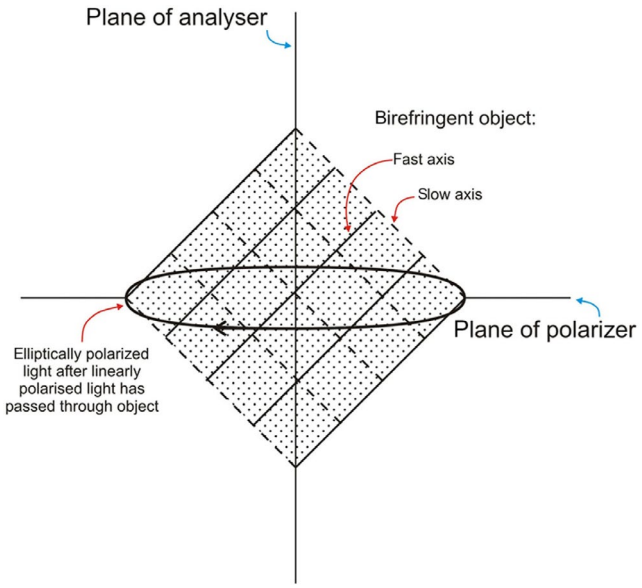

FIGURE 3 Diagram to show that linearly polarized light coming towards the observer, passing through a birefringent object with its fast and slow axes at 45° to the polarizer plane, is converted to elliptically polarized light, some of which can pass a crossed analyser, making the object appear bright against a dark background. The size of the ellipse in the plane of the analyser depends on the retardance, which is the thickness of the object multiplied by the birefringence. The direction of rotation of the ellipse depends on the relation between the fast and slow axes and the polarizer plane. Reproduced from *Bull R C Pathol*. **144**, 263-266 (2008), with permission

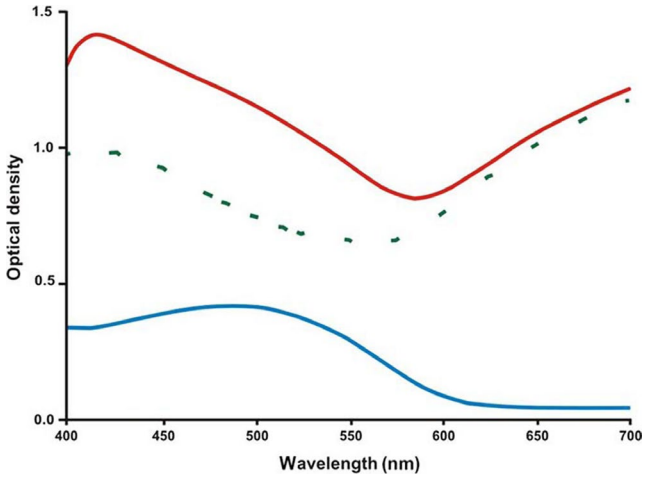

FIGURE 4 Measured absorbance, expressed as optical density, of Congo red orientated at 45° between crossed polarizer and analyser (red line) at different wavelengths of light. This is the net result of transmittance of light expected from birefringence (green interrupted line) and reduction of transmittance produced by absorbance of Congo red at 45° to the polarizer plane (blue line). Reproduced from *Lab Invest*. **88**, 232-242 (2008), with permission

chemist Oskar Wälchli.[38,39] Neubert, using smears of Congo red, confirmed Ambronn's finding that the orientated dye was dichroic, and showed that it was also birefringent, giving a bright yellow colour. He investigated the explanation of this. It was known that the brightness of a birefringent material was related to the retardance, which is the thickness of the material multiplied by the amount of birefringence, measured as the difference between the refractive indices of the slow and fast axes. The retardance equals the distance between the tip of the wave vector in the fast axis and that of the vector in the slow axis as they emerge into air. The size of the ellipse in the plane of the analyser, and so the theoretically transmitted brightness, ranges from nil, meaning that no light of that wavelength can pass the analyser, to maximal, meaning that there is potentially most transmission by the analyser. When the retardance is expressed in terms of wavelengths, there is no transmission if there is no birefringence or if the retardance is a whole wavelength or a multiple of it, and there should be most transmission when the retardance is half a wavelength, or a wavelength and a half, and so on (Figure 3).

Neubert measured the retardance of Congo red at five wavelengths of light and found that this was maximal in yellow light but nil in blue light. He postulated that this meant that blue light would not be transmitted by a crossed analyser, and so the transmitted colour would be white lacking blue, which is yellow. Neubert realized correctly that the transmitted colour would be modified by absorption of some light by Congo red. This is because in any birefringent, absorbing material, such as Congo red, orientated with the fast and slow axes at 45° to the plane of linearly polarized light, there is still some absorption, the amount of which is halfway between the dichroic maximum and minimum. The interaction of absorption and birefringence in the transmittance of light by Congo red was confirmed and measured in 2008, although Neubert's report of yellow in smears of Congo red and his claim of no birefringence in blue light are contrary to later experience (Figure 4).[3]

Neubert showed that the birefringence of Congo red varied with wavelength, but made no attempt to explain the reason for this. According to him, all birefringence was positive. Positive birefringence means that the slow axis is parallel to a recognizable feature in the birefringent material, in this case the direction of smearing of Congo red, but which in other cases could be, for example, the long axis of crystals or of amyloid fibrils. Negative birefringence is when the slow axis is perpendicular to the recognizable feature.

An important paper by the German chemist Hans Zocher later in 1925 not only corrected this mistake by Neubert, but also explained how birefringence varied with wavelength, although Zocher's clear account which gave the physical principles underlying many of the later findings in Congo red-stained amyloid was rarely quoted.[40] Zocher used established theories to consider any dichroic material. This absorbs some wavelengths of light polarized parallel to one orientation, but not light polarized perpendicularly. A non-absorbing material was known to show a slight decline in refractive index as

wavelength increased, called normal dispersion of the refractive index. An absorbing material, in contrast, was known to show a sharp change in refractive index around an absorption peak. The index is minimal on the immediate shortwave side of a peak, and maximal on the longwave side. This is called anomalous dispersion of the refractive index (Figure 5).

In a dichroic material, the non-absorbing plane has normal dispersion, while the absorbing plane has anomalous dispersion. The birefringence, or difference between the refractive indices of the planes, is largest around an absorption peak, which explains how birefringence varies with wavelength. The birefringence also changes sign around a peak, because the slow and fast axes exchange orientations around a peak (Figure 5). In this way, Zocher corrected Neubert's claim that Congo red showed only positive birefringence.

Zocher and his colleague Friedrich C. Jacoby gave more detailed evidence for the theory of the variation of birefringence with wavelength in an account of smears of nearly two hundred dyes, including Congo red.[41] They confirmed that in Congo red, as in most dyes, absorption is strongest of light polarized parallel to smears, and so its birefringence is negative at shorter wavelengths below the absorption peak, which is in the blue-green, and positive at longer wavelengths above the peak. This was confirmed and measured in 2008 (Figure 6).[3] The position of the fast and slow axes in relation to the polarizer plane determines the direction of rotation of the elliptically polarized light produced by a birefringent material, which can be clockwise or anticlockwise. If the axes exchange positions, the direction of rotation reverses (Figure 3). This can be achieved either by the effect of anomalous dispersion at different wavelengths as the sign of birefringence changes around an absorption peak, or by rotation of any birefringent material through 90°.

The birefringence of Congo red-stained amyloid was discovered in 1927 and published in French. Paul Divry (1889-1967) was a Belgian psychiatrist whose histochemical study of cerebral plaques in dementing diseases was a by-product of his research on cerebral lipids with his student, Marcel Florkin (1900-1979), who was later a distinguished biochemist. Divry, working with formalin-fixed frozen sections, found that unstained plaques were weakly birefringent, and that staining with Lugol's iodine increased the birefringence. The staining with iodine led him to conclude that the cores of plaques were amyloid. He confirmed this by staining with metachromatic dyes.[42,43]

Divry knew Bennhold's work and showed that the cores of plaques stained with Congo red. He mentioned as an aside during proof correction that Congo red, like iodine, increased

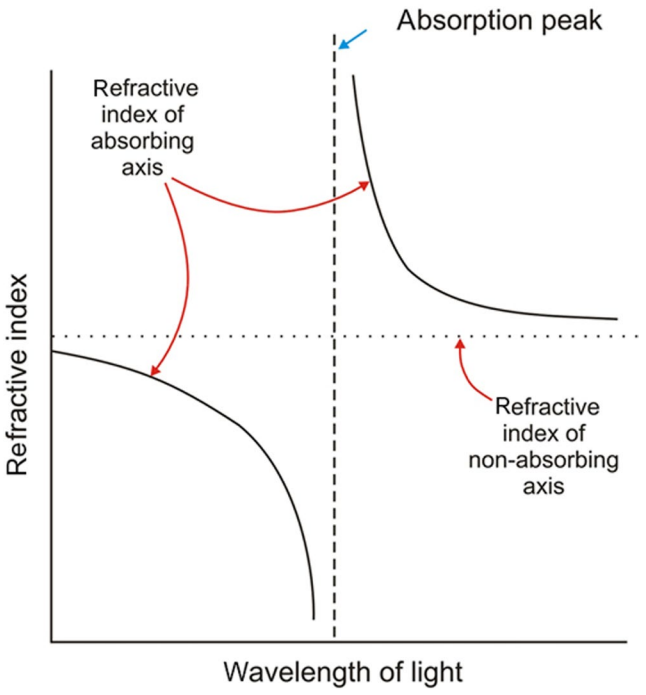

FIGURE 5 Relation between the refractive index and wavelength of light in the absorbing and non-absorbing axes of a dichroic material, such as orientated Congo red. There is anomalous dispersion of the refractive index around an absorption peak in the absorbing axis. The refractive index falls to its lowest value on the immediate shortwave side of the peak and jumps to its highest value on the immediate longwave side of the peak. Meanwhile, the refractive index is relatively constant in the non-absorbing axis. As a result, the birefringence, which is the difference between the refractive indices of the axes, is not only largest around the absorption peak, but also changes sign, because the higher refractive index is in the non-absorbing axis at wavelengths below the peak, and in the absorbing axis at wavelengths above the peak. Reproduced from *Bull R C Pathol*. **144**, 263-266 (2008), with permission

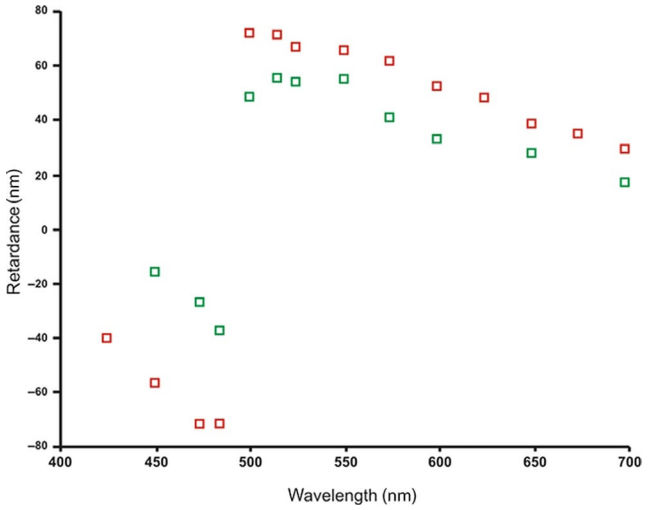

FIGURE 6 Measurements of the retardance of smears of Congo red (red squares) and Congo red-stained amyloid (green squares) at different wavelengths of light. Absolute retardance is maximal around the absorption peak of Congo red, at about 500 nm, with a change from negative retardance at wavelengths below the peak to positive retardance at wavelengths above the peak. Reproduced from *Lab Invest*. **88**, 232-242 (2008), with permission

the birefringence of the cores of plaques, that is amyloid. Divry did not illustrate this in his first paper, but in his paper with Florkin, in which they applied the various staining methods to amyloidosis of the spleen and experimental amyloidosis, there is a monochrome figure showing birefringence in amyloidosis of the spleen stained by Congo red. Divry and Florkin said that they were studying the mechanism of the optical properties of amyloid with a crystallographer, but they do not appear to have published anything on this.[44]

Claims have repeatedly been made that Divry[42] or Divry and Florkin[44] reported a green or apple-green colour under these conditions.[17,36,45-59] This is an example of how mistaken attributions can be passed from paper to paper. In fact, Divry never reported a colour in Congo red-stained amyloid examined between crossed polarizer and analyser, even in the final summary of his work.[60]

4 | FIRST REPORT OF COLOURS IN CONGO RED-STAINED AMYLOID

The credit for recognizing that there are colours is due to Paul Ladewig (1909-1992), who emphasized his priority.[61] He was a German pathologist who worked in Istanbul, Turkey, from 1935, and then moved to the USA in 1946.[62] His work appears independent, because his paper, published in 1945 in English, has no references.[63] Perhaps because he was independent, he had no expectations of what he ought to see, and simply and accurately reported his findings. This should also have applied to Divry and Romhányi, who must have seen colours but did not mention them. George Romhányi (1905-1991), a Hungarian pathologist, apparently independently of Divry, reported at a meeting in 1942, published in 1943 in German, that Congo red-stained amyloid was birefringent. Romhányi did not say that he saw any colours until 1971, long after others had done so, when he only mentioned green, without an explanation of the mechanism.[64-70]

Ladewig found that Congo red-stained amyloid changed twice from yellow to green during 360° rotation of a slide, although the illustration is in monochrome. "Yellow and green" is an incomplete description of the range of possible colours in Congo red-stained amyloid, but the mixture is certainly a common finding (Figure 1). Ladewig did not explain the colours, but they indicate that his microscope did not have perfect optics.

The optics is not perfect when there are components in the light path that have unwanted birefringence. In an ideal optical system, in which only the Congo red-stained amyloid is birefringent, there is green between accurately crossed polarizer and analyser. This is blended from blue and yellow, because every green shade can be matched by an appropriate mixture of blue and yellow. Blue is the net colour transmitted by the analyser as a result of negative birefringence of wavelengths below the absorption peak of Congo red, modified by absorption. Yellow is the net transmitted colour as a result of positive birefringence of wavelengths above the absorption peak, modified by absorption (Figure 2).

Most microscopes have accidental birefringence in the light path, which is strain birefringence from stressed glass in slides, coverslips or lenses. The elliptical light produced by these can convert elliptically polarized light produced by Congo red molecules to linearly polarized light in the plane of the polarizer, which means the light cannot be passed by the crossed analyser. This is called compensation and is only effective when the ellipses have opposite directions of rotation, and the light in the ellipses has the same wavelengths. For some investigations, for example, measurement of retardance, birefringence of known orientation and variable strength can be deliberately introduced into the light path by use of a device called an elliptical compensator. The results of accidental or deliberate additional birefringence are that yellow ellipses can be partially or completely compensated, converting green to blue-green or even blue, and blue ellipses can be similarly compensated, giving yellow-green or even yellow.

The outcome is that rather than pure green, a mixture of blue-green and yellow-green, or even blue and yellow, may be seen at different sites in Congo red-stained amyloid, depending on the relative strengths and orientations of the birefringence of the Congo red molecules and the strain birefringence (Figures 1 and 2). A pair of colours exchange positions when the section is rotated by 90°, because the direction of rotation of the ellipses from the Congo red changes but that from strain birefringence of lenses keeps the same relation to the polarizer. Ladewig had strain birefringence in his microscope and saw yellow-green and blue-green, which he called yellow and green, and at any point, the colours changed at every 90° rotation, although Ladewig did not note that as they changed they passed through black. This is the position at which the slow and fast axes are parallel or perpendicular to the polarizer and analyser planes and cannot give birefringent effects, which require a light wave to have vectors in both axes. Accordingly, the effects are maximal when the axes are at 45° to the polarizer plane.

In the previous section, "Early polarization microscopic observations," Neubert[38] was noted to report only a bright yellow colour in smears of Congo red between crossed polarizer and analyser. Neubert must have had strain birefringence in his microscope, which compensated negative birefringence, removing transmission of blue. This explains how he saw yellow in smears, rather than green, and how he found no birefringence in blue light and only positive birefringence, unlike later findings.[3,40,41] With compensation, smears, because the Congo red molecules are orientated in parallel, show only one colour at a time, rather than the two shown in different parts of sections of Congo red-stained amyloid

with randomly orientated fibrils, as Ladewig[63] found. Also, with compensation, smears change colour if they are rotated by 90°.[3] Neubert must have studied smears all at a consistent orientation in relation to the polarizer and analyser, because otherwise he would have seen a blue colour at times.

If Ladewig[63] had uncrossed the polarizer and analyser, he would have seen other pairs of colours. This is because as the filters are progressively uncrossed, birefringent effects decline, the background becomes lighter, and orientated Congo red approaches either its darkest red from maximal absorption or its lightest red from minimal absorption, depending on the relation between the orientation of the Congo red and the rotated filter. The various colours seen are a blend of the progressively declining birefringent colour or colours with the progressively increasing dichroic colours, and are always multiple, even in perfect optical conditions. At different sites, pure green becomes either light blue-green, then bright white, and finally a dull, neutral, colourless appearance, or yellow, orange, bright red and finally dull red. Initially, blue-green and yellow-green, or blue and yellow, may show virtually any mixture of colours, depending on the orientation of the amyloid fibrils and the direction of rotation of the polarizer. This was shown in 2008.[3]

The adjective to be applied to the colours that are different from the red of Congo red-stained amyloid under ordinary illumination is anomalous. Anomalous colours are well known in crystallography but not in biological microscopy.[71] The mechanism that explains them differs from other processes postulated to give the colours in Congo red-stained amyloid. Interference colours, such as those seen in soap bubbles, have been suggested, but require much larger retardances, or differences in the phase of waves, than those ever measured in sections of Congo red-stained amyloid.[15,38,49,54,60,72-75] Detection of interference colours between crossed polarizer and analyser requires retardances of whole wavelengths, but reported retardances in Congo red-stained amyloid have invariably been under half of any wavelength.[4] Optical rotation, which is rotation of the plane of linearly polarized light by optically active substances such as sucrose in solution, has also been suggested as a mechanism.[53,76] This is negligible in sections of Congo red-stained amyloid, although this can be shown in solutions with a much greater optical path length than that through histological sections.[4]

5 | PURE GREEN MAKES ITS APPEARANCE

Hans-Peter Missmahl (1920-2008) was responsible almost singlehandedly for the idea that green, and only green, is essential for the diagnosis of amyloid. He qualified in medicine in 1947 in Tübingen and for a dissertation was encouraged by the pathologist Erich Letterer (1895-1982) to study amyloid.[77] In 1949, Missmahl became a physician under Bennhold, who had moved from Hamburg to Tübingen in 1942. In 1969, Missmahl moved the opposite way to become director of a medical clinic in Hamburg and retired in 1985.[78]

Missmahl's first paper on amyloid in 1950 was his dissertation and reported histochemical findings.[79] Like most of his publications, this was written in German. He was aware of the work of Romhányi and Ladewig and used polarization microscopy, but did not mention any birefringent colours. In his paper in 1953 with Marga Hartwig (born 1921), who was a physician in the Medical Clinic of the University of Tübingen from 1950 to 1953, Missmahl described only a green colour in Congo red-stained amyloid between crossed analyser and polarizer. Missmahl and Hartwig called the green an anomalous absorption colour, although they did not explain what this meant. They mentioned Ladewig, but not his description of yellow and green colours.[80] In 1955, Missmahl noted that the German microscopist Hans H. Pfeiffer in 1953 had reported yellow and green polarization colours in Congo red-stained amyloid in tissue culture, but Missmahl insisted that only green should be seen if the microscopic optics are perfectly correct, which is true.[35,81]

Missmahl gave further findings in 1957, including that birefringence was stronger in green light than in red.[45] He never explained why this should be so, only saying, "The basis for the green polarisation colour is therefore the strong dispersion of the birefringence of the Congo red with a maximum in green light".[82]

Missmahl did not draw any conclusions about the fine structure of amyloid from the optical properties of Congo red-stained amyloid and proposed that the properties were attributable to the deposition of amyloid on pre-existing connective tissue fibres, apparently because this idea was suggested to him by Letterer.[77,80] This led to several publications by Missmahl on two supposedly different types of amyloid, peri-reticular and peri-collagenous.[83-87] This idea never became popular.[28,88] Romhányi corrected this view and established that the properties were due to an ordered, micellar structure of amyloid itself, which he and Ladewig had suggested before Missmahl's connective tissue idea.[65-67] Missmahl later claimed that he had predicted the fibrillary structure of amyloid in 1953 and 1957.[83,84]

Like Neubert and Wälchli, Missmahl thought that orientated Congo red showed only positive birefringence.[45,48,80,89,90] This is inconsistent with anomalous dispersion both in theory and in practice, but Missmahl never indicated that he was aware of this theory, and never quoted Zocher[40] or Zocher and Jacoby,[41] who showed how the birefringence of Congo red changes sign around its absorption peak (Figures 5 and 6). Strangely, in a later work Missmahl knew that toluidine blue had a change of sign of birefringence around its absorption peak, which had been shown by the Welsh pathologist Douglas B. Brewer (1919-2016).[91] Brewer

proved that toluidine blue absorbed light polarized at right angles to its orientation, which is unlike most dyes, including Congo red.[41] Missmahl said that the graph of birefringence of toluidine blue differed greatly from that of Congo red but did not realize that his concept of only positive birefringence of Congo red was wrong, nor did he try to explain the dispersion curve of toluidine blue.[90,92]

Although Missmahl included a graph to show the positive birefringence of Congo red in a few of his later publications, it seems to have been based on a figure in a paper in 1959 by the German pathologists Paul Bernd Diezel and Albrecht Pfleiderer,[47] because if it were Missmahl's own work, he gave no reference, nor did he give any details of his method or findings.[48,86,89,90] Diezel and Pfleiderer measured the retardance of Congo red-stained amyloid at several wavelengths and reported only positive birefringence, maximal around the absorption peak, although they gave no explanation of this. They seemed unfamiliar with the theory of anomalous dispersion and must have assumed that all their measured retardances were positive.

Diezel and Pfleiderer showed changes of colour from green through yellow and orange to red as the thickness of sections increased. The explanation, not given by them but detailed and confirmed later, is that both absorption and birefringence contribute to the transmitted colour, and although the effects of both increase as section thickness increases, absorption predominates and progressively removes more blue and green from the transmitted colour.[3,93] Like Ladewig and Pfeiffer, in sections of the usual thickness, Diezel and Pfleiderer saw green and yellow between crossed polarizer and analyser, which they called, wrongly, "yellow green dichroism," and attempted to explain, implausibly, by different thicknesses of Congo red in the yellow and green areas, rather than by the more feasible effects of strain birefringence (Figure 1).

Missmahl knew that strain birefringence gave yellow and green, and that uncrossing the polarizer and analyser gave green and red, but he did not mention any other colours such as blue or orange, and his observations on these changes were faulty. His explanations were also faulty and show how his understanding of the physical optics was incomplete. A few times he attempted to account for the effects of strain birefringence, but never mentioned elliptically polarized light and did not explain compensation properly.

For example, he wrote, "Rotation of the elliptic compensator during illumination with white light first compensates the weak birefringence of red and blue light. The weak components of red and blue light which accompany the green anomalous colour are thereby extinguished. This causes the intensification of the anomalous green light of clockwise rotation of the compensator. Counterclockwise rotation of the compensator does not cancel the double refraction of the embedded Congo red, but brightens the field of view, thus enhancing the intensity of the white light. This causes an increasing yellow color upon counterclockwise rotation".[90]

This account misunderstands the mechanism of compensation. Missmahl does not say that in the first case the colour turns progressively from green to blue by compensation of yellow, and thinks that both ends of the spectrum are compensated. In the second case, he thinks that no colours are compensated but somehow the yellow appears not by compensation of blue but by intensification of the background white colour, which actually happens whichever way the compensator is turned, but is only slight (Figure 2).

He postulated that there was no absorption of light by thin sections or smears, which was why the green colour was seen between crossed polarizer and analyser, unaffected by absorption. This is not only wrong from experimental evidence (Figure 4), but is easily disproved by observation, because if there were no absorption, sections and smears would be colourless in ordinary illumination.

As evidence of his misunderstanding of the interaction of absorption and birefringence, he wrote, "In the first example (rotation of the polariser towards the right) we bring the vibration plane of the light coming from the polariser and arriving onto the preparation in the direction in which the pigment particles strongly absorb green light…. This entails the disappearance of the green anomalous polarisation colour and the appearance of the red absorption colour of the dye. In the second example (rotation of the polariser to the left), the vibration plane of the light arriving onto the preparation is on the contrary turned more in the direction in which the pigment only absorbs a little green light. The green anomalous polarisation colour therefore remains".[82]

In fact, birefringent effects decline equally whichever way the polarizer is turned, and Missmahl did not describe the colour changes accurately as birefringence declined and absorption either increased or decreased.

6 | MISSMAHL'S INFLUENCE ON THE USE OF "GREEN BIREFRINGENCE"

Despite the findings of Divry, Romhányi, Ladewig, Missmahl and Pfeiffer, there was initially little use of polarization microscopy in the routine study of amyloid, even as Congo red became more popular as a stain. Highman in 1946 did not mention polarization microscopy, nor did contemporary and some later texts on staining methods.[30,94] In 1956, Symmers wrote that the birefringence of Congo red-stained amyloid was inconstant and of no diagnostic value, and this was repeated in texts.[95,96] Missmahl had an important influence on the use of polarization microscopy in the study of Congo red-stained amyloid, although the introduction of this into routine practice, even his own, took time. Until 1962, Missmahl used

Bennhold's intravenous Congo red test to diagnose amyloid, and only after that began to use rectal biopsies and Congo red staining when he suspected amyloid.[86]

Papers from the mid-1950s onwards gradually began to report the use of polarization microscopy, and usually referred to Missmahl and Hartwig[80] or Missmahl[45] or both. For the study of Congo red-stained amyloid, Missmahl insisted that a microscope specifically made for polarization microscopy was essential, and that an ordinary microscope with a polarizer and an analyser was unsuitable.[6,82,86,89,97] It is doubtful whether much notice was taken of this requirement, which is unrealistic in everyday practice and moreover is unnecessary. Because most observers used an ordinary microscope, there was almost always strain birefringence in the optical system, and so anomalous colours as well as green appeared. Despite this, it was unusual for authors to report colours other than green. As a rare but confused example, a paper in 1963 mentioned "positive birefringence and dichroism (green changing to pinkish orange)".[98]

The insistence on green, and green alone, for the diagnosis of amyloid can be traced to the influence of Missmahl. He consistently used only "green", although his expressions varied, and included "green polarisation colour," "green anomalous polarisation colour," sometimes described as "specific," and "characteristic green birefringence".[80,82,83,86,89,99] The first use of "green" alone in polarization microscopy in a paper in English appeared to be in 1959 by the American physician Alan S. Cohen (1926-2018) and his colleagues, who quoted Missmahl and Hartwig[80] and Missmahl[45].[100] The significance of the qualification "in English" is that apart from the 1945 paper by Ladewig,[63] whose "yellow and green" colours were almost entirely ignored, all the publications before 1959 on the colours seen on polarization microscopy of Congo red-stained amyloid, and the few that attempted explanations of them, were in German. Furthermore, many were in obscure journals. These factors alone, apart from the difficulty of finding a simple account of the relevant optical principles, would have dissuaded many workers from consulting the original papers and would have persuaded them to take second-hand statements on trust.

The German histochemist Holde Puchtler (1920-2006), who moved to the USA in 1955, published a couple of influential papers in 1962 and 1964.[31,101] In the first of these, she introduced her improved Congo red staining method, but she did not specify a colour in the "polarization microscopic properties" in either paper. Afterwards, "green" became almost invariable by the early 1970s. This was in a variety of expressions, such as "green polarization colour," or "green birefringence," sometimes qualified by "characteristic," "classical," "specific," "typical" and "unique," or "green anomalous colour," or, incorrectly, "green dichroism".[28,49-51,53,72,74,102-105] A well-known text in 1968 instructed that "examination by polarized light must always be carried out," and used Missmahl[45] as a reference for the "absolutely specific green anisotropic colour".[106]

Confirmation and reinforcement of Missmahl's role in the supposed need for "green birefringence" in the diagnosis of amyloid came in the first international symposium on amyloidosis in Groningen in 1967, in one of Missmahl's few published presentations in English. Notable people in amyloid research were there, including Cohen and the American pathologist George G. Glenner (1928-1995), who separately wrote important reviews on amyloid, disseminating ideas in the English-speaking world.[28,55,107] In his 1967 review, Cohen, discussing the "unique green birefringence," wrote, "This is the single most useful histologic test for amyloid." At the symposium, he said, "Again, I would guess that the consensus of the meeting is that in the diagnosis of amyloidosis the use of an appropriate biopsy and the Congo red stain followed by polarization microscopy to show green birefringence is probably the best method we have to date." Cohen also said, "Other people have discovered and rediscovered the birefringence, but it was really Dr Missmahl who brought to the forefront the significance of the green birefringence in making the diagnosis of amyloid".[88]

In the symposium, Missmahl talked about polarization microscopy and said, "An ordinary light microscope with polarizer and analyser is not necessarily a polarization microscope," and, "If a change in colour is observed [on rotating the section] the quality of the microscope is not good enough for investigation of amyloid deposits." He criticized other presenters, saying to one, for example, "I think the slides have demonstrated that he does not have a real [polarisation] microscope, because if you see green and yellow in one picture, or, as in one of your slides, green and red, then your microscope is wrong!… The optical system must be absolutely strain free".[86]

Cohen's comments emphasize Missmahl's powerful influence on the dissemination of the idea that green was essential for the diagnosis, and Missmahl's comments reinforce his view that other colours should not be seen, or if they are, that they show that there is supposedly something wrong with the microscope and should not be reported. Missmahl's comment about seeing green and yellow was unfortunately rather undermined by the fact that in an earlier paper he had included a few colour figures that showed definite green and yellow.[2,83]

7 | THE APPEARANCE OF "APPLE-GREEN BIREFRINGENCE"

"Apple-green" first appeared in the USA in the mid-1950s as a description of the colour seen in fluorescein on immunofluorescence microscopy of materials other than amyloid. "Green" had been the original description.[108-110]

"Apple-green" began to be applied to the colour of Congo red-stained amyloid in the early 1970s, apparently by spread from its use in fluorescence. An example of how the transfer could have occurred, by close apposition of the two descriptions, is shown by the use of "apple-green" to describe fluorescence findings in the 1967 amyloidosis symposium, in which polarization microscopic findings on Congo red-stained amyloid were only described as "green".[111] Similarly, a paper on amyloid in 1973 used both "typical green birefringence of Congo red" and "apple green fluorescence of thioflavine".[112] How the two concepts could easily be confused is shown by the repeatedly mistaken use of terms such as "green fluorescence under polarized light" and "apple green fluorescence under polarized light microscopy" to describe the properties of Congo red-stained amyloid, when "fluorescence" was written instead of "birefringence".[77,113-115]

The first paper using "apple-green birefringence" of Congo red-stained amyloid seems to have been published in 1972 in the United Kingdom by Australian authors.[116] An editorial in 1973 may have helped to disseminate the term, which it had copied from a paper under discussion, published in the United Kingdom in 1973 but written in 1972.[117,118] Like these other publications, a chapter, written in 1972 and published in 1973, did not give a reference to support the use of "apple-green".[119] "Apple-green birefringence," and even the unquestionably erroneous "apple-green dichroism," began to appear in textbooks, which both reflected their increasing use and helped their dissemination.[54,120]

"Green" and "apple-green" eventually seemed equally popular.[2] Even Cohen, who favoured "green," occasionally used "apple-green," and later wrote that "Congophilia with apple green birefringence was the first criterion of amyloid to be adopted".[121,122] Glenner also favoured "green" but occasionally used "emerald-green".[55,107]

8 | THE ORTHODOXY OF "GREEN BIREFRINGENCE" QUESTIONED

Hardly anyone criticized Missmahl's dogmatic view. One was Philip Schwartz (1894-1977), a neuropathologist born in Hungary, who moved to the USA in 1953. For the diagnosis of amyloid, he favoured fluorescence with thioflavine S, which Missmahl discounted, commenting at the 1967 symposium, "I am a little afraid to say that the thioflavine method is specific. I think you have a lot of side effects".[86] Here and elsewhere, Schwartz said about Congo red on polarization microscopy, "Quite often, however, this green color combined with other hues…. You see a senile plaque stained with Congo red, then the same senile plaque in polarized light. Indeed, Missmahl's green is right there. But you realize that the largest part of the deposited mass does not display Missmahl's color; it is yellowish and also brown, although definitely amyloidotic".[7,123]

Missmahl replied, "I have written 13 years ago that such plaques are amyloid and in my old pictures all the plaques show real green birefringence color," referring to Missmahl and Hartwig.[34,86] Another who cautiously questioned Missmahl's rule was the English biomedical scientist Robert J. Francis, who wrote in a chapter, "In the author's experience, it is difficult even with high quality optical apparatus completely to exclude some yellow birefringence of amyloid deposits".[119]

Despite the evidence of everyday practical experience, which shows that in Congo red-stained amyloid between crossed polarizer and analyser, it is unusual to see pure green, and it is common to see no green at all, few observers have queried whether "green birefringence" is the correct term.[2] Several things seem to have contributed to this acceptance of the orthodox belief. One is that uncritical repetition in papers, texts, meetings, lectures and clinical practice has made people use the term automatically, and even if they saw other colours, they assumed that green had been proved to be essential for the diagnosis of amyloid.

Another factor is limited knowledge of the underlying physical optical principles, which have been given at appropriate points in this paper but are not widely known and are difficult to find explained all at the same time in a non-specialized way. These principles are neglected in medical texts, but can be found scattered through advanced reference works in physics or polarization microscopy, which are not consulted in routine medical practice.[71,124-126] Virtually all microscopists are familiar with birefringence in the sense that they know that it means that a material appears bright between crossed polarizer and analyser, but most would probably not be able to explain the brightness. Fewer still would be able to explain the colours seen.

Apart from Zocher,[40] hardly any workers investigating the optical properties of Congo red or other materials showed evidence that they were familiar with anomalous dispersion of the refractive index, although this is a property of all light-transmitting substances (Figure 5). Zocher himself did not describe anomalous colours in Congo red.[40,41] Perutz and Mitchison used the theory of anomalous dispersion in an explanation of the anomalous colours seen in crystals of reduced haemoglobin.[127] Similarly, Brewer explained anomalous colours in various dyes.[91] Brewer and colleagues later investigated Congo red and confirmed and explained the anomalous colours, including the interaction of birefringence and absorption.[3]

Although the physical optics of Congo red-stained amyloid is now understood, the orthodox view of "green birefringence" has been established for so long that it is understandably difficult to alter. One step in this direction was that the Nomenclature Committee of the International Society of Amyloidosis suggested in 2014, and confirmed in 2016, that the diagnosis of

amyloid requires "green, orange or yellow birefringence" to be seen, which is a change from its previous requirement of "green birefringence".[1,128,129] Unfortunately, the Nomenclature Committee reverted to "green-yellow birefringence" and "yellow-green birefringence" in 2018.[130]

9 | CONCLUSIONS

There were long gaps between the introduction of Congo red as a histological stain in 1886 and the realization in 1922 that it detected amyloid, between the detection of the birefringence of Congo red-stained amyloid in 1927 and the report of colours in 1945, and between the insistence on green in 1953 and the description and explanation of the range of anomalous colours in 2008. The insistence that green alone should be seen, and is essential for the diagnosis, mainly arose from the idea that microscopic optics had to be perfect, which they rarely are in practice, and also from an incomplete understanding of the optical principles. Green does not have to be seen on its own or even mixed with other colours to indicate that Congo red molecules are orientated on a material, and the usual material that does this in routine medical practice is amyloid.

The history of Congo red-stained amyloid illustrates a few unscientific aspects of medicine, such as how dogmatic statements based on misunderstanding of physical principles may not be questioned and are accepted as facts. Moreover, the misconceptions and misquotations passed from paper to paper give an example of inappropriate referencing and citation distortion, the dangers of which include that "Erroneous and unfounded claims can be perpetuated, which sets back real scientific progress".[131,132] The history also shows, even more strikingly, how observation is not objective but is influenced by the expectation of seeing what common opinion believes should be seen.

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